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PROVISIONAL APPLICATION COVER SHEET

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This is a request for filing a PROVISIONAL APPLICATION as defined and under 37 C.F.R. §1.9(a)(2) and §1.53(c).

Attorney Docket Nº: CSURF-107P

Inventor/Applicant:

Susan P. James, Ph.D.
46 Redstone Lane,
Bellvue, CO 80512
Citizenship: USA

Min Zhang, M.S.
1500 West Plum Street, #8D,
Fort Collins, CO 80521
Citizenship: PR China

Title: **HA ESTERIFICATION VIA ACYLATION TECHNIQUE FOR MOLDABLE DEVICES**

Direct all correspondence to:

Customer Number: **28304**

Jean M. Macheledt / Tel. (970) 218-6798
Macheledt Bales LLP
501 Skysail Lane, Suite B100, Fort Collins, CO 80525-3133

Enclosed Application Parts:

X Specification and technical description — *Number of Pages:* 8
X 3 sheet of figures — FIG. 1 and FIG. 2A - 2E
X Attachments (A1, A2 - C) — *Number of Attachments* five (each copied as *single-sided*)

✓A1 (5 pgs.) / A Graphs and Table (2 pgs.) / A2 (1 pgs) / B (1 pg.) / C (6 pgs.)

Assignee: **Colorado State University Research Foundation, Fort Collins, Colorado USA**

Applicants and Assignee, Colorado State University Research Foundation, claim SMALL ENTITY STATUS under 37 CFR § 1.27(a)(1) and (3) – Person/individual and Nonprofit Organization.

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Respectfully Submitted as authorized,

Signature: _____ Date Signed: 19 th September 2003

Printed Name: Jean M. Macheledt, Patent Attorney Reg. No. 33,956

PROVISIONAL APPLICATION FILING ONLY

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CSURF No. CSU-04-007 *mbj* Atty Docket CSURF-107P (*as filed – 19th September 2003*)
Inventors: Susan P. James, Ph.D. and Min Zhang, M.S.

Hyaluronan (HA) Esterification via Acylation Technique for Moldable Devices

REFERENCE TO PRIOR APPLICATION(S)

This provisional application relates to technology referenced in pending U.S. nonprovisional patent application no. 10/283,760 filed on behalf of the assignee hereof on 29 Oct 2002. Both the technology disclosed in the instant application and that of U.S. nonprovisional patent application no. 10/283,760—and its predecessor provisional application no. 60/340,777 (filed October 2001), were developed while applicants were under an obligation of assignment to the assignee hereof.

BACKGROUND OF THE INVENTION

In general, the present invention relates to the synthesis and use of hyaluronan (a.k.a., hyaluronic acid, sodium hyaluronate), and other hydrophilic polymers with pendant hydroxy groups that are not generally melt processable in their native state, in connection with medical devices such as temporary and permanent implants, surgery instruments and aids, as well as other mechanisms (whether considered biocompatible) that is entirely composed of or have a member or component made of hyaluronan, or other hydrophilic polymers with pendant hydroxy groups that are not generally melt processable in their native state,

As further explained by applicants in **ATTACHMENT A1** and the **Graphs and Summary Table** therefor and in **ATTACHMENT A2** (all of which are fully incorporated herein by reference) and as supported and depicted in the flow diagram labeled FIG. 1 and by FIGs. 2A – 2E depicting example representative chemical structures, of particular interest, here, is the synthesis of a novel moldable hydrophilic polymer—preferably, hyaluronic acid (HA)—produced in a manner that lowers its melting point below the point at which the polymer degrades such that it is no longer useful for an application. Pure (whether synthetic or genetically engineered or native) HA, for example, has a melting point above the point of substantial degradation making it by-and-large impossible to mold HA into structures suitable for use. A polymeric material produced according to the unique technique of the invention, provides a melt processable polymer (*e.g.*, HA) that,

once re-hardened (*i.e.*, cooled or solidified), can be used in a variety of product applications, whether resultant structures are used 'as is' having been molded, extruded, or otherwise shaped into a piece/device/etc. or employed as a component or subassembly of an assembly/ system.

Structure(s) produced according to the invention may be composed entirely of the melt processable derivatized HA (or other polymer with hydroxy groups) and used alone, or used as scaffold for biological materials (*e.g.*, cells, morphogenic proteins) or incorporated into a component, piece, module, feature, or any structure/member to produce a 'system' such that the HA's hydrophilic outer surface provided is interior- or exterior-facing, *etc.* A non-exhaustive list of possibilities contemplated hereby for the generally hydrophilic outer surface produced atop the novel outer layer, include: a bearing surface (for various items such as gears, fishing rod eyelets, bearings of all types, joint and other weight-bearing mechanisms, whether incorporated as part of manufacturing equipment, as part of the manufactured product itself, *etc.*); a flexible barrier surface separating a first and second area (such flexible barriers to include the membrane material or tubing used for catheter balloons, catheter tubing, hot air balloons, condoms, IV tubing, diaphragms, flexible bladders, *etc.*); a transparent member surface (such members to include the transparent planar or curved polymeric films and sheet material used where optical clarity is sought such as for fish tanks, polymeric covers for vehicle, water- or aircraft head-lamps and blinkers/fog-lights, covers for spot-lights, windows on or in a vehicle, aircraft, watercraft, and spacecraft, monitor and television screens, ophthalmic lenses, camera lenses and view-finders, *etc.*); an *in vivo* implant surface (any of a variety of total or partial joint replacements, splints, stents, diaphragms, *etc.*); a drag reduction surface (for components of a vehicle, watercraft, aircraft and spacecraft such as hulls, pontoons, vehicle-body parts, blades/runners, *etc.*, as well as the glide-surface of snowboards, water and snow skis, and so on); a reaction resin surface (such as research or industrial use components); a topical dressing surface (such dressings to include, without limitation, those used for medical/veterinary applications such as adhesive bandages, sterile pads for wounds and surgical procedures, bandage tape/adhesive, ace bandages, soft casts, *etc.*); and a dental splint surface (such splints to include mouth-guards, tooth/jaw-correction splints, *etc.*).

Additional contemplated applications of this technology include any HA (or other hydrophilic polymer with hydroxy groups that is not generally melt processable)-based biomaterials that would benefit from a net-shape molding process. Furthermore, the derivatization of the HA permits control of its hydrophobicity, expanding the range of useful solvents and non-solvents during manufacturing and possibly allowing tailoring of the biological activity of HA. Examples of such HA biomaterial applications include: tissue engineered scaffolds for cartilage and other biological tissue repair and treatment, wound dressings, artificial skin, viscoelastics for intra-surgical protection and prevention of post-operative adhesions, hydrophilic, lubricious and/or anti-fouling and/or anti-coagulant coatings (e.g., catheters, contact lenses, dialysis membranes), drug release/delivery devices, and biodegradeable materials (e.g., nerve guides). In the spirit and scope of design goals contemplated hereby, the novel melt processed structures produced according to the invention may be made by chemically modifying a wide variety of hydrophilic polymers containing pendant hydroxy groups, including without limitation: poylsaccharides, polyalcohols, glycoaminoglycans, polyhydroxy polymers and cellulose.

As one will appreciate, prior use(s) by others of HA, for example that identified by the applicants of **ATTACHMENT C** (paragraphs reproduced below for handy reference), focus on creating chemically modified HA for use as pharmaceuticals, foodstuffs, cosmetics (e.g., moisturizing agent or lotion) and other flowable, gel-like substances. This focus causes the methods employed to be different than that according to the invention:

> [0050] The chemically modified hyaluronate according to the invention is characterized in that the reduction in molecular weight thereof during the preparation process is considerably decreased in comparison with the modified hyaluronates prepared by the processes of the prior art. The average molecular weight of the modified hyaluronate prepared by the present invention varies depending on an average molecular weight of a hyaluronate as a raw material and/or the reaction conditions. For instance, where a hyaluronate having an average molecular weight of 500,000 to 1,500,000 is employed as a raw material, the average molecular weight of the modified hyaluronate of the invention is a not less than approximately 20%, preferably not less than 30% of the average molecular weight of the raw material.
>
> [0051] The chemically modified hyaluronates according to the present invention are usable as a material for the pharmaceuticals, the foodstuffs, the cosmetics or the like, but not limited thereto. They are particularly useful for the medical application; for example as a moisturizing agent, a smoothing agent, a wound covering material, a material of drug delivery system (DDS) or the like, because the chemically modified hyaluronates which are prepared by the nonaqueous reaction contain a reduced amount of the contaminants such as a pyrogen, an antigenic substance or the like and are metabolizable in the body. Especially, it is expected that the degradation rate of the modified hyaluronates in uterus highly correlates with the biorhythm of the human body or uterus and thus the modified hyaluronates according the present invention are significantly useful as a device for intrauterine or intervaginal transplantation material which supports a therapeutic agent, e.g. an agent for endometriosis.

BRIEF DESCRIPTION OF ATTACHMENTS AND FIGURES

For purposes of illustrating the innovative nature plus the flexibility of design and versatility of the technique and resulting material of the invention the following background references and figures have been included. One can readily appreciate the advantages and the many features that distinguish the instant invention from conventional methods. The figures as well as the following technical manuscript authored by the applicants hereof, and additional materials, have been included to communicate the features of applicants' innovative process and material by way of example, only, and are *in no way* intended to unduly limit the disclosure hereof. Each identified enclosure is labeled an **ATTACHMENT** and is hereby fully incorporated herein by reference; accordingly each listed **ATTACHMENT** is described below:

[A1] Min Zhang, Ph.D. candidate and Susan P. James, Ph.D., a confidential internal manuscript ___ pages in length and which remains confidential, included herewith for its discussion of features of the invention and background information; incorporated herein in further support of the novel features of the invention.

Graphs and Summary Table for ATTACHMENT A1 included for its technical information in connection with **ATTACHMENTS A1 and A2**, representing features and characteristics of an example material produced according to the invention.

[A2] Min Zhang, *et al.*, confidential manuscript 1 page in length as confidentially submitted by the applicants hereof and upon the filing hereof, to the 7th World Biomaterials Congress (May 17-21, 2004 in Sydney, Australia); included herewith for its discussion of features of the invention and as background information in further support of the invention.

[B] US Patent Application no. 10/283,760 filed on behalf of the assignee hereof for the applicants on 29 Oct 2002, published to James *et al.* 1 May 2003 as US 2003/0083433 A1; cover page hereof enclosed, with the full application incorporated herein by reference as technical background support.

[C] Patent Application No. US 2002/0143171 A1 published 3 October 2002 to Yui *et al.*; 6 pages thereof included herewith for its background technical reference information, only.

FIG. 1 is a flow diagram depicting details of a process 10 of producing structure(s) according to the invention—illustrated in FIG. 1 are not only core features, but also further distinguishing features of the invention employing chemical structures such as those structures represented and depicted, by way of example only, in FIGs. 2A – 2E.

DESCRIPTION DETAILING FEATURES OF THE INVENTION

As can be better appreciated by viewing FIG. 1 and associated representative example chemical structures, and as outlined by applicants in their **ATTACHMENTS A1** and **A2** (which also reference features depicted in FIGs. 1 and 2A – 2E), a further summary of the core features of process 10, follows. For purposes of support of the representative example discussed throughout, FIGs. 2A – 2E depict various molecular structures employed in connection with producing structures according to the invention.

As shown: the chemical formula for (plain) hyaluronic acid (HA) at 20 in FIG. 2A; in FIG. 2B the formula for cetyltrimethylammonium bromide 22, as is the structure of the salt cetylpyridinium chloride monohydrate 24; in FIG. 2C a polymeric complex represented by the expression $HA^- - QN^+$ is shown and labeled 26, where HA represents hyaluronic acid and QN^+ represents a cation; in FIG. 2D a silylated $HA^- - QN^+$ complex is depicted after having performed step 14, FIG. 1; and in FIG. 2E the silylated complex of FIG. 2D has been acylated according to the invention to produce the melt processable polymer after having performed step 16, FIG. 1. As one can appreciate (FIG. 2D), the cloaking of $HA^- - QN^+$ complexes results in the hydrophilic groups being replace with silylated functional groups: hydrogen (H) has been replace with $Si(CH_3)_3$; and once an acylation of the FIG. 2D polymer complexes using an acid chloride (preferably one having a hydrocarbon tail of sufficient length such that the complexes have an opportunity to become melt processable) has been performed (step 16, FIG. 1), a complex such as that depicted in FIG. 2E results. The example represented by FIG. 2E is a silylated HA^--QN^+ complex [*i.e.* H having been replaced with -$Si(CH_3)_3$] that has been acylated such that its -$Si(CH_3)_3$ groups are now replaced with $-CO(CH_2)_{10}CH_3$ – as shown. As pointed out in **ATTACHMENTS A1** and **A2**, many acylation agents are contemplated hereby.

As may be used, for reference, several acronyms and structures are identified as listed:

BSA – N, O-Bis(trimethylsilyl)acetamide, a silylation agent.
DMF – N, N-Dimethyl formamide
DMSO – Dimethyl sulphoxide
HA – Hyaluronic acid
HA-CPC – the complex of HA polyannion and cetylpyridinium salt.
HA-CTAB – the complex of HA polyannion and cetyltrimethylammonium salt.
HA^- –QN^+ – the complex of HA polyannion and long-chain paraffin ammonium cation.
HMDS – hexamethyldisilazane, a silylation agent.
THF – Tetrahydrofuran
TMCS – trimethylchlorosilane, a silylation agent.
QN^+ – long-chain paraffin ammonium cation.

Returning to FIG. 1, a method 10 of producing structure(s)/devices/product according to the invention is illustrated—as mentioned, included are core, as well as further distinguishing, features of the invention. Steps of the method include: Produce complexes of a hydrophilic polymer such as HA (box 12). Temporarily cloak (*e.g.*, via silylation) at least a portion of the hydrophilic groups of the HA-quat. ammonium salt complex. Perform esterification employing suitable process(es) such as acylation of the polymer complexes using an acid chloride (box 16). As noted in FIG. 1 (box 16) acylation happens at silyl groups as that is where the acid chloride 'attacks' the FIG. 2D complex(es) such that the hydroxyl groups of the silylated HA are typically more reactive than the hydroxyl groups on non-silylated HA. A desired shape is attained by thermal/thermo-processing employing any of a number of suitable process whereby the lower melt temperature may be attained and pressure sufficient to produce the end-structure(s)/ device(s)/piece(s) (box 17). Remove the acyl groups from the polymer complexes in solution, e.g., alkaline solution (Box 32). to produce a generally hydrophilic outer surface of the outer layer. Return the polymer to a pre-complex state (e.g., in NaCl solution to recover hydrophilic groups (box 34). The thermally formed structure(s)/piece(s)/etc. may be used 'as is' and further processed as necessary (box 39a) or further processed and built into an assembly (box 39b)—depending upon final application of the end-product.

As mentioned, while only HA has been showcased here, a variety of hydrophilic polymers for use to carry out the unique features of the method of the invention, are contemplated; these may be obtained off-the-shelf and include new materials yet to be developed for distribution for commercial and/or research/analytical purposes.

EXAMPLE 1. Also referenced in applicants 29 October 2002 pending utility application (see the published application herein labeled **ATTACHMENT B** for further reference)—by way of example the following is offered: In the case of using HA, which is strongly hydrophilic with its many polar groups (-COOH, -OH and $-CONHCH_3$) on its long molecular chain. Therefore a modification of the HA molecules is done to increase hydrophobicity and compatibility with organic solvents used in connection with acylation.

1A) Silylation of HA to increase its hydrophobicity: Silylation is a known technique for increasing hydrophobicity, and createorganic-soluble derivatives of substances. During a silylation reaction of HA, the hydrophilic groups containing active

hydrogen, such as –COOH, -OH, and $-NH_2$, are masked by hydrophobic silyl groups. The reaction is reversible, the silylated functional groups can be returned to their original state through hydrolysis reaction. HA is a muco-polysaccharide of molecular weight up to millions ($\sim 10^6$). Compared with silylation of poly-L-lysine (MW = $\sim$ 1000), silylating HA is difficult due to its large molecular weight. In contrast to PLL silylation previously performed by the applicants (see **ATTACHMENT B**), preferably HA is modified before silylation to increase its solubility in silylation solvents (polar organic solvents can be used). The steps include:

(1) Reaction of HA with long-chain aliphatic quaternary ammonium salts (QN^+). Polyanions, such as HA, combined with certain organic cations, such as paraffin chain ammonium (QN^+) ions, produces a precipitable complex. The complex is a true salt of the polyacid and quaternary base. HA was modified with long-chain aliphatic ammonium salts, to improve its solubility in organic solvents. Combination of QN+ with polyannions occurs in those pH ranges in which the polyannions are negatively charged. The reaction between HA and ammonium cations in water can be expressed:

$$HA^-\text{-}Na^+ + QN^+A^- \rightarrow HA^-\text{-}QN^+ \downarrow + Na^+ A^-$$

where $HA^-\text{-}Na^+$ is the sodium salt of hyaluronic acid; $HA^-\text{-}QN^+$ is the precipitable complex between HA carboxylic polyanion and long chain paraffin ammonium cations. $HA^-\text{-}QN^+$ (HA-CPC / HA-CTAB) complexes were used. The complexes ($HA^-\text{-}QN^+$) precipitated from HA aqueous solution are soluble in concentrated salt solutions, so HA can be recovered from its insoluble complexes. Ammonium salts used were: cetyltrimethylammonium bromide monohydrate (MW: 358.01) (CTAB) and cetylpyridinum chloride (M.W. 364.46) (CPC).

(2) Silylation of $HA^-\text{-}QN^+$ complexes: HA-CPC and HA-CTAB were silylated in DMSO solution with BSA, HMDS and other typical silylation agents. Silylation agents are generally sensitive to humidity, silylating operation should be under the purge of dry N_2.

1B) Acylation of HA to improve its thermal flow: To make HA flowable at high temperature, the strong hydrogen bonding between its molecules must be disrupted, and

the molecular order (i.e., crystallinity) of HA needs to be destroyed. Acylating the hydroxyl groups on HA with long-chain aliphatic carboxylic acids chloride will help in de-crystallizing HA. Acid chlorides, from caproyl to stearoyl chloride, can be used as acylating agents. Acylation is a known process for disrupting crystallinity in other polysaccharides. Acylation reactions are performed in solution (of HA^-- QN^+ in DMSO, for example). Start with a DMSO solution of HA^-- QN^+ complex using the technique described above in connection with Silylation of HA, above. Acylation is done to make the hydrophilic polymer melt processable.

While certain representative embodiments and details of an example have been shown merely for the purpose of illustrating the unique technique and associated melt processable material and structure(s) of the invention, those skilled in the art will readily appreciated that various modifications may be made to representative embodiments without departing from the novel teachings or scope of this technical disclosure. Accordingly, all such modifications are intended as contemplated to be included within the scope hereof. Although the commonly employed preamble phrase "comprising the steps of" may be used herein, or hereafter, in a method claim, the applicants do not intend to invoke 35 U.S.C. Section 112 §6. Furthermore, in any claim that is filed (herein for illustrative purposes as well as claims added hereafter in a utility application), any means-plus-function clauses used, or later found to be present, are intended to cover the structures described herein as performing the recited function and not only structural equivalents but *also* equivalent structures.

HA Esterification via Acylation Technique for Moldable Devices
Internal TECHNICAL DISCLOSURE ADDENDUM

Min Zhang, Ph.D. candidate, Mechanical Engineering
Susan P. James, Ph.D. Associate Professor, Mechanical Engineering
Colorado State University

INTRODUCTION: The technology described herein is related to the patent application filed October 29, 2002 entitled "Outer Layer having Entanglement of Hydrophobic Polymer Host and Hydrophilic Polymer Guest". Our October 29, 2002 patent application details background technical characteristics and qualities of hyaluronic acid (HA) and discusses a multitude and variety of product applications for which that product may be used. This technical discussion represents a new acylated HA moldable material. In particular, in this disclosure addendum we describe an improved, updated polymer technology that embodies a further unique modification and use of HA such that it can be melted and molded, thermo-processed, or otherwise shaped, into useable forms for structures for which the biocompatibility, biological and/and other characteristics are a factor. In our pending patent application filed October 29, 2002, the process of creating HA esters through acylation was described in connection with the synthesis of a novel outer layer having an entanglement of at least a hydrophobic polymer host and a hydrophilic guest, wherein molecules of the guest are crosslinked through primary bonding (*e.g.*, chemical/covalent cross-linking). The unique layer in our 2002 pending application, as fabricated, has a generally hydrophilic outer surface useful in a wide variety of applications when laminated, thermally or otherwise bonded, formed, or integrated with a base member or structure comprising a hydrophobic polymer. Thus, discussion in our 2002 pending patent application focused on a way to modify a hydrophilic guest material, such as HA, so that it may be used with a hydrophobic host adapted for use as a base or substrate (important for its structural support) for molding into a shape or laminating/molding/bonding or otherwise adhering to another structure

Here, surprisingly, we've found that the HA esterification process in and of itself, is useful and suitable for a wide variety of products that can be molded from the HA without requiring employing a hydrophobic host material to operate as a structural support for the material.

Unmodified HA, and the currently available commercial derivatives thereof, do not melt and flow without degradation to the material, and thus cannot be molded into product. One reason for this is that the degradation temperature of HA and its derivatives is lower than its melting temperature due to strong intermolecular hydrogen bonds. To make HA flowable at a lower temperature (i.e., moldable), the strong hydrogen bonding among its molecules must be disrupted -- the molecular order of HA needs to be disrupted. Acylating the hydroxyl groups on HA with long-chain aliphatic carboxylic acid chloride achieves this. The resultant esterified HA can be thermally melted (i.e., its melting temperature is below its molding temperature). The one specific example (details below) which we have reduced to practice involves the use of lauroyl chloride (acyl radical: $CH_3(CH_2)_{10}CO$-) as an acylation agent. The resultant esterified HA has a melting range of 70-105° C with a peak melting endotherm at 93° C (from DSC in air with a heating rate of 10°C/min). The degradation of that compound (from thermal gravimetric analysis in air with a heating rate of 10°C/min) begins at 190° C (providing a large window of approximately 90° C between melting and degradation for safe processing of the polymer). While heating in air at a higher rate (20° C/min) under a microscope the same

esterified HA compound showed visual evidence of melting at approximately 100° C with no signs of concurrent degradation.

Fidia, S.p.A. (Padova, Italy) has received certain patents on esterified HA. The HA esters obtained through our method are different from Fidia's. Fidia's patents all relate to esterification of HA carboxyl groups with alcohol [Valle, 1989]. None of Fidia's HA esters can be molded because they degrade before they melt.

METHODS: In the present process, acid chlorides, from caproyl (i.e., hexanoyl) to stearoyl chloride, can be used as acylating agents, although the shorter carbon chain acyl radicals may not sufficiently decrease the melting point below the degradation point (see ANALYSIS below). Acylation is often used for other polysaccharides [Kurita, 2001], but to the best of our knowledge no one has used this process to modify HA.

The starting material is silyl HA-CTA (see our earlier filed, 29 October 2002, pending patent application and earlier work for how this product is made) which is HA (MW: ~1.5 million Daltons, Genzyme, Cambridge, MA) that has had the active hydrogens of the hydroxyl groups replaced with trimethylsilyl groups ($-Si(CH_3)_3$), In the most recent work, hexamethyldisilazane or HMDS was used as the silylation agent in the solvent dimethyl sulfoxide (DMSO) at 75° C for 24 hours. Prior to silylation the HA was complexed with a long-chain aliphatic ammonium salt (cetyltrimethylammonium bromide w/ MW: 358.01, or CTA) to improve its solubility in DMSO.

During esterification, the trimethylsilyl groups [$-Si(CH_3)_3$] attached to the oxygen of hydroxyl groups were substituted by acyl groups. The reaction was performed without solvent medium. The procedure used was as follows:

(1) 200mg of silyl HA-CTA was added under dry nitrogen to 5-10 equivalents of acid chloride (e.g., lauroyl or hexanoyl to date) at room temperature. The mixture was gently heated to the holding temperature (80-100 C).
(2) The liquid mixture was heated for 1 hr at 80-100C. A clear solution with light brown color was obtained, but once cooled, the solution became turbid.
(3) Removed the excess acid chloride [the by-product chlorotrimethylsilane (b.p. 57C) was completely distilled off during reaction].
    a. The acid chloride with low boiling point (such as hexanoyl chloride, bp. 151-153C) was evaporated under vacuum in a 60-80C water bath.
    b. For the acid chloride with high boiling point (such as lauroyl chloride, b.p. 134-137C/under 11 mm high mercury column), HA-ester was precipitated from the resulting solution with a non-solvent for the HA-ester and a solvent for the acid chloride (such as hexane for lauroyl chloride).
(4) Vacuumed the above product at 60C until the weight was constant.
(5) HA esters recovered were light brown particles.

DSC and TG Analysis: The thermal properties of both (hexanoyl and lauroyl) HA esters made to date were determined using a Seiko DSC SCC 2200 differential scanning calorimeter (DSC) and a Seiko TG SCC 5200 thermal gravimetric analyzer (TGA) at a heating rate of 10°C/min in air.

Hot Stage Microscope Analysis: The HA ester film samples were prepared by evaporating solutions of HA ester in organic solvents: 1,2-dichloroethane was used for the hexanoyl HA ester, and a mixture of xylenes and DMSO was used for the lauroyl HA ester. The HA ester films were placed between a microscope slide and a cover slip. The slide was placed on a hot stage that was mounted on a Nikon polarizing light microscope. The polymer was heated at a rate of 20°C/min and optical photomicrographs were taken under crossed polarizers with a Nikon FX-35DX camera.

Referenced herein and below, is the 2 page attachment entitled **Graphs and Summary Table for: ATTACHMENT A...** ; there are two graphical representations, one labeled Figure 1 and the other Figure 2, and a **SUMMARY TABLE** which provides information about "thermal properties of HA esters made with different acyl radicals" as labeled.

RESULTS/ANALYSIS: DSC thermograms for both HA esters (hexanoyl and lauroyl) are shown in Figure 1. The thermogram for the lauroyl ester shows a broad melting endotherm from 70°C to 105°C with a peak at 93°C, while the melting endotherm of the hexanoyl ester ranges from 175°C to 218°C with the peak at 190°C. TG analysis (Figure 2) shows that the HA hexanoyl ester begins degrading at about 170°C, while the lauroyl ester begins degrading at about 190°C. Optical photomicrographs under for both esters were taken between room temperature and 200°C. Melting liquid droplets became obvious in the lauroyl ester at about 100°C. The hexanoyl ester did not appear to begin melting until 190°C, but at this temperature the liquid also turned brownish in color, evidence of the onset degradation. The fact that the onset of melting observed under the microscope coincided with the endotherm peaks found in the corresponding DSC thermograms, but not with the endotherm onsets, may be due to the much higher heating rate used in hot-stage and the difficulty of seeing the first-formed liquid in the photo pictures. The table at the end summarizes the thermal properties of the two different esters. The lauroyl ester clearly melts well before it begins to degrade.

DISCUSSION: The above results demonstrate that HA can be converted to a thermally meltable and flowable material through esterification with appropriate acid chlorides. On each disaccharide unit of HA, there are four hydroxyl groups, one amide and one carboxyl group. Clearly, the introduction of aliphatic acyl groups at the hydroxyl groups disrupts the intermolecular bonds, reducing the crystallinity and bringing about appreciable thermoplasticization. In other words, the acyl groups act as internal plasticization agents, as observed in esterified cellulose produced by a similar acylation method [Shiraishi et al, 1979]. The higher aliphatic acid chloride (lauroyl) was more effective in conferring thermoplasticity to HA than the lower aliphatic acid chloride (hexanoyl). The HA ester derived from the lauroyl radical ($CH_3(CH_2)_{10}CO$-) can melt without any accompanying degradation. The hexanoyl group ($CH_3(CH_2)_4CO$-) was too small to sufficiently disturb the molecular order of HA, so the HA hexanoyl ester did not achieve thermal fluidity before the degradation. Future work will analyze the resultant compounds, explore the effectiveness of intermediate-sized acyl radicals at enhancing the melt-processability of HA esters, and examine how completely an HA ester molded to

final shape can be converted back to HA. The acyl groups can be easily removed in alkaline solution through saponification. Surface hydrophilicity will be measured before and after saponification, with the expected result that hydrophilicity will increase to that of unmodified HA after saponification. Finally, future work will examine how well the degree of esterification (and resultant thermal properties) can be also controlled by controlling the degree of silylation in the starting material. In conclusion, the HA lauroyl esters produced by aclyation of the hydroxyl groups on HA have a melting temperature considerably below their degradation temperature and thus should be melt processable. These HA esters are different from those patented by Fidia Advanced Biopolymers (Italy), on which the carboxyl groups were esterified with alcohols [Valle, 1989].

REFERENCES:

1. Valle, della, Esters of Hyaluronic Acid, US Patent 4851521, Jul. 1989 – this is one of Fidia's patents. See US Patent 5,336,767 (also della Valle) as another example.
2. Kurita, K., Controlled Functionalization of the Polysaccharide Chitin, Progress in Polymer Science, Vol. 26, pp.1921-1971, 2001.
3. Nobuo Shiraishi, Tadayo Matsunaga, and Tokuo Yokota, Thermal Softening and Melying of Esterified Wood Prepared in an N_2O_4-DMF Cellulose Solvent Medium, Journal of Applied Polymer Science, Vol. 24, 2361-2368, 1979.

Comments regarding published Patent Application US 2002/2014371 (Yui, Nobuhiko):

We have created a chemical modified HA by 0-acylating a complex of HA and a cationic compound. The cationic compound we prefer is a quaternary ammonium salt. We perform acylation to make HA moldable (drop its melting temp below its degradation temp). Preferably acylation is done with an acid chloride that has a significant hydrocarbon tail. While the Yui patent application starts with a complex of HA and a quat. ammonium salt and then acylates this in an nonqueous solvent, we begin much differently.

By way of example, we start with an HA-quat. ammonium salt complex, and then silylate this complex, and then acylated the silylHA-quat. ammonium complex. The silylation predictably improves the acylation result because acylation happens at the silyl groups (the acid chloride attacks there). Goal is to get a more complete silylation and/or have easier reaction conditions (lower T, time, no solvent) than without the silylation. Also the byproduct in this case is TMCS (trimethylchlorosilane or $(CH_3)_3SiCl$), which is easily removed. Without silylation the byproduct would be HCl. Our acylation reaction does not use (or need) an acid binder, as Yui does, because we do not have an acidic byproduct (HCl).

Preferably we do not use a solvent for this reaction and the Yui patent uses a nonaqueous solvent. The acid chloride used in our work is in liquid form and is the carrier for the reaction. The sily-HA-quat ammonium complex is dissolved in the acid chloride. The lack of a solvent has potential biocompatibility benefits because there is less concern about residual solvents. This may also enhance reactivity during acylation (no dilution).

We prefer the use of bigger acid chlorides to aid in lowering the melting point of HA below its degradation temperature. The hydrocarbon tail on the acid chloride interferes with intermolecular bonding, decreasing the melting temperature. When we used hexanoyl chloride, the resultant HA derivative had a melting temperature just below (or about equal to) its degradation temperature.

All acid halides, including the chlorides, may be employed as acylation agent (one example being acid chloride). For example, the acid chloride Lauroyl Chloride is useful as it has 10 CH_2 groups (for a total of 12 carbons) in its hydrocarbon tail. Hexanoyl has only 4 CH_2 groups (for a total of 6 carbons) and does not decrease the melting temperature enough. All the compounds in between these two (with 6 and 8 CH_2 units in the hydrocarbon chain) are contemplated to work also, as well as acid chlorides with even more than lauroyl, such as those with 14 and 16 CH_2 units in the tail. The structure of these is shown below, by way of example:

Hexanoyl chloride : $CH_3(CH_2)_4COCl$
Octanoyl chloride: $CH_3(CH_2)_6COCl$
Decanoyl chloride: $CH_3(CH_2)_8COCl$
Lauroyl Chloride: $CH_3(CH_2)_{10}COCl$ – (did drop degr. Temp well below melt temp.)
Palmyroyl chloride: $CH_3(CH_2)_{14}COCl$
Stearoyl Chloride: $CH_3(CH_2)_{16}COCl$

Attachment A2 to Provisional Application Attorney Docket CSURF-107P filed 19-Sep-03

Modification of Hyalur nan to make it Moldable and to Control its Hydrophilicity

Zhang, M.; Wheeler, D.; James, SP
Colorado State University, Fort Collins, CO, USA

INTRODUCTION: The clinical uses of hyaluronan (HA) (a.k.a., hyaluronic acid, sodium hyaluronate, etc.) continue to expand. Applications of HA-based biomaterials include tissue engineered scaffolds, intra-surgical protection and prevention of post-operative adhesions. A significant challenge to the practical production and use of HA based biomaterials is that HA and the currently available commercial derivatives thereof, cannot be molded because their melting points are above their degradation temperatures. Furthermore, the extreme hydrophilicity of HA limits its solubility in nonaqueous solutions, making it difficult to create HA structures in conjunction with more hydrophobic materials. The objective of this study was to create a novel HA ester with improved hydrophobicity that is melt processable, and that can easily be returned to the unesterified state after processing.

METHODS: Synthesis of HA Ester [patent pending]: (1) Sodium HA (MW: ~ 1.5 million Daltons, Genzyme) was complexed with a long-chain quaternary ammonium cation, cetyltrimethylammonium (CTA) bromide (MW: 358.01). The complex (HA-CTA), which was precipitated from aqueous solution, is soluble in dimethyl sulfoxide (DMSO). (2) The active hydrogens of the hydroxyl groups on HA were replaced with trimethylsilyl groups [-$Si(CH_3)_3$]. The reaction was carried out in DMSO (silylation grade, Pierce) under dry N_2 at 75°C for 24 hrs. The starting product was the HA-CTA and the silylating agent was hexamethyldisilazane (HMDS, Aldrich). HMDS does not dissolve in DMSO, so the final product was a two-phase solution. Silyl HA-CTA with a high degree of substitution (DS) was in the HMDS layer, while that with a low DS was in the DMSO layer. The HMDS was evaporated at 60°C under vacuum. The resulting silyl HA-CTA product could be dissolved in hexane, xylenes, toluene, tetrahydrofuran, etc and was the starting material for synthesis of the HA esters. (3) HA hexanoyl ester and lauroyl ester were synthesized through acylation of the -$OSi(CH_3)_3$ using acid chlorides. 200 mg of silyl HA-CTA were added under dry nitrogen to 5-10 equivalents of hexanoyl ($CH_3(CH_2)_4COCl$) or lauroyl ($CH_3(CH_2)_{10}COCl$) chloride liquid (Aldrich). The mixture was heated for 1 hr at 80°C. A clear, light brown solution was obtained, but when cooled, the solution became turbid. The excess hexanoyl chloride was removed under vacuum at 65°C. The HA lauroyl ester was precipitated from the excess lauroyl chloride with hexane. The precipitate was washed with hexane several times, and then dried under vacuum. DSC and TG Analysis: The thermal properties of both HA esters were determined using a Seiko DSC SCC 2200 differential scanning calorimeter and a Seiko TG SCC 5200 thermal gravimeter at a heating rate of 10°C/min in air. Hot Stage Microscope Analysis: HA ester films were cast from organic solvents (1,2-dichloroethane for hexanoyl ester, and xylenes/DMSO for lauroyl ester) and placed between a microscope slide and a cover slip. The slide was placed on a hot stage that was mounted on a Nikon polarizing light microscope. The polymer was heated at a rate of 20°C/min from room temperature to 200°C.

RESULTS: The DSC thermograms for both HA esters are shown in Figure 1. The thermogram for the HA lauroyl ester shows a broad melting endotherm from 70°C to 105°C with a peak at 93°C, while the melting endotherm of the HA hexanoyl ester ranges from 175°C to 218°C with the peak at 190°C. The TG analysis (Figure 2) shows that the HA lauroyl ester begins degrading at about 190°C, while the HA hexanoyl ester begins degrading at approximately 170°C. Optical photomicrographs of the HA lauroyl ester are shown in Figure 3. Melting liquid droplets became obvious at approximately 100°C. On the hot stage the HA hexanoyl ester did not appear to melt until 190°C, but at this temperature the liquid also exhibited the brownish appearance typical of degradation. The melting onsets determined using the hot-stage microscope coincided with the peaks of the endotherms in the corresponding DSC thermograms, but not with the onsets. This is probably due to the much higher heating rate used in the hot-stage, and the difficulty of detecting the first-formed liquid drops in the micrographs.

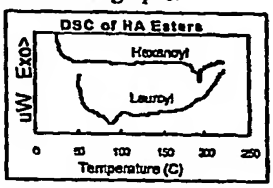


Figure 1: DSC of HA esters

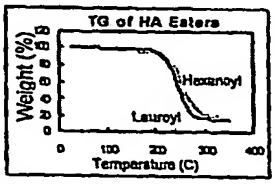
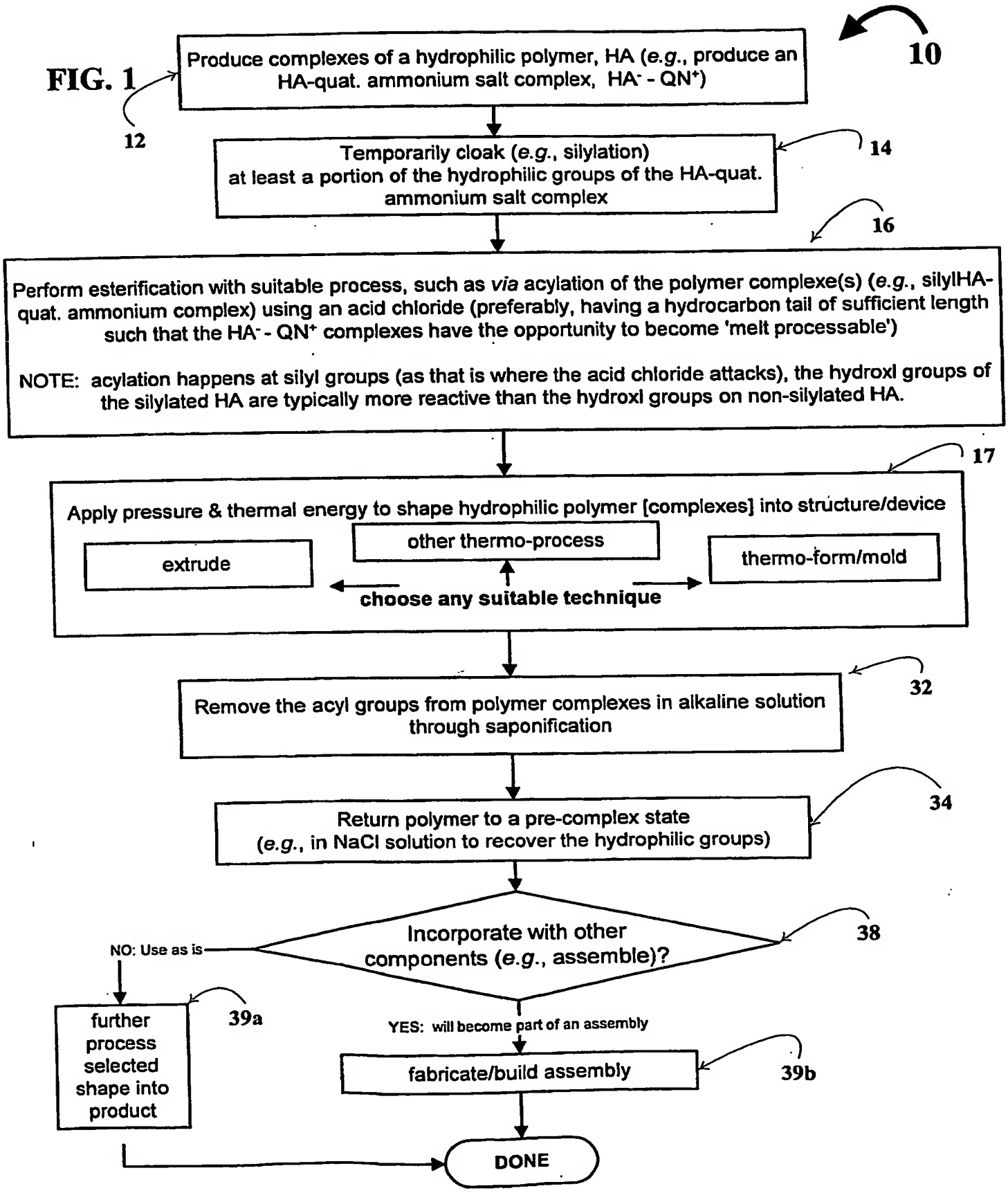


Figure 2: TG of HA esters

Figure 3: Optical micrographs of HA lauroyl ester under crossed polarizers at 100°C (left), and 160°C (right).

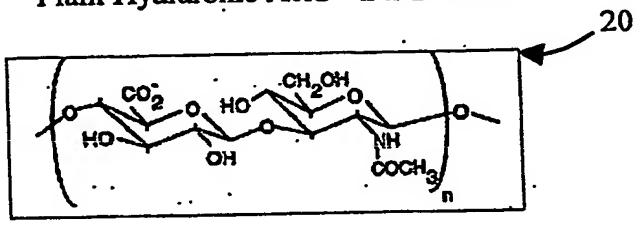
DISCUSSION AND CONCLUSIONS: This study demonstrates that HA can be converted to a thermally melting and flowable material through esterification with appropriate acid chlorides. The introduction of aliphatic acyl groups disrupts the strong intermolecular bonding, reducing the crystallinity and bringing about appreciable thermoplasticization, similar to that observed in esterified cellulose [Nobuo et al, J. App. Polym. Sci, Vol. 24, 2361-2368, 1979.]. The higher aliphatic acid chloride was more effective in conferring thermoplasticity to HA. The HA lauroyl ester has a melting range of 70-105°C and degradation of the compound does not begin until 190°C, providing a large window for safe processing of the polymer. The hexanoyl acyl radical was not sufficiently large enough to disturb the molecular order of HA, so the HA hexanoyl ester did not achieve complete thermal fluidity before the onset of degradation. The acyl groups can be easily be removed from the HA esters via saponification. Soaking in NaCl solution will remove any remaining silyl or CTA groups.

FIG. 1

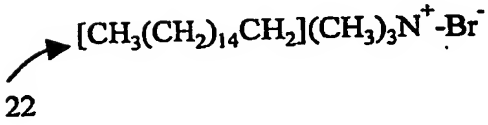

10
Produce complexes of a hydrophilic polymer, HA (e.g., produce an HA-quat. ammonium salt complex, HA⁻ - QN⁺)
12
Temporarily cloak (e.g., silylation) at least a portion of the hydrophilic groups of the HA-quat. ammonium salt complex
14
16
Perform esterification with suitable process, such as via acylation of the polymer complexe(s) (e.g., silylHA-quat. ammonium complex) using an acid chloride (preferably, having a hydrocarbon tail of sufficient length such that the HA⁻ - QN⁺ complexes have the opportunity to become 'melt processable')
NOTE: acylation happens at silyl groups (as that is where the acid chloride attacks), the hydroxl groups of the silylated HA are typically more reactive than the hydroxl groups on non-silylated HA.
17
Apply pressure & thermal energy to shape hydrophilic polymer [complexes] into structure/device
extrude
other thermo-process
thermo-form/mold
choose any suitable technique
32
Remove the acyl groups from polymer complexes in alkaline solution through saponification
34
Return polymer to a pre-complex state (e.g., in NaCl solution to recover the hydrophilic groups)
38
Incorporate with other components (e.g., assemble)?
NO: Use as is
39a
further process selected shape into product
YES: will become part of an assembly
fabricate/build assembly
39b
DONE

HA – Hyaluronic acid
HA-CPC – the complex of HA polyannion and cetylpyridinium salt.
HA-CTAB – the complex of HA polyannion and cetyltrimethylammonium salt.
HA^- –QN^+ – the complex of HA polyannion and long-chain paraffin ammonium cation.
HMDS – hexamethyldisilazane, a silylation agent.
THF – Tetrahydrofuran
TMCS – trimethylchlorosilane, a silylation agent.
QN^+ – long-chain paraffin ammonium cation.

Plain Hyaluronic Acid **FIG. 2A**



Cetyltrimethylammonium Bromide



Cetylpyridinium Chloride Monohydrate

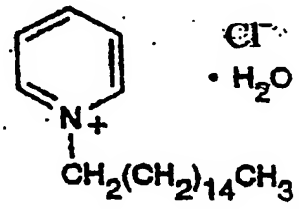
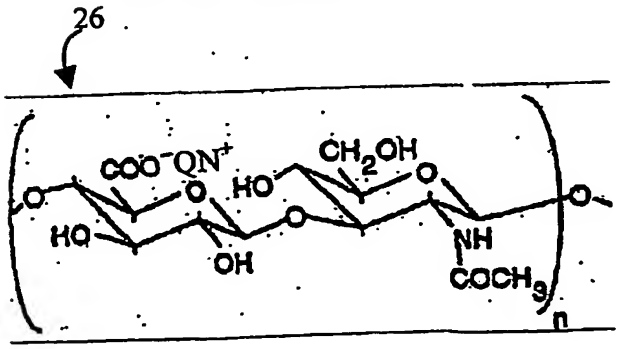


FIG. 2B

HA^--QN^+ complex **FIG. 2C**



Silylated HA^--QN^+ complex **FIG. 2D**

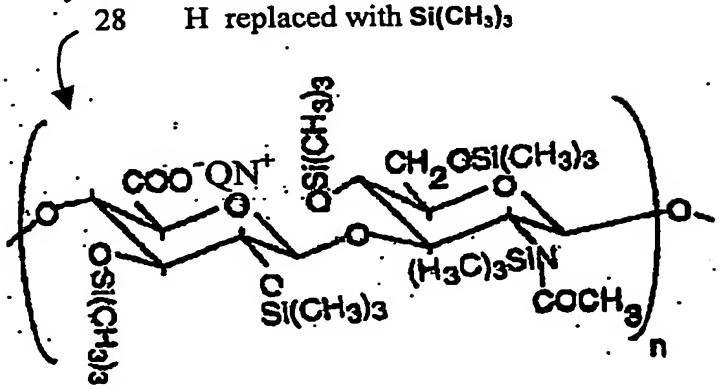
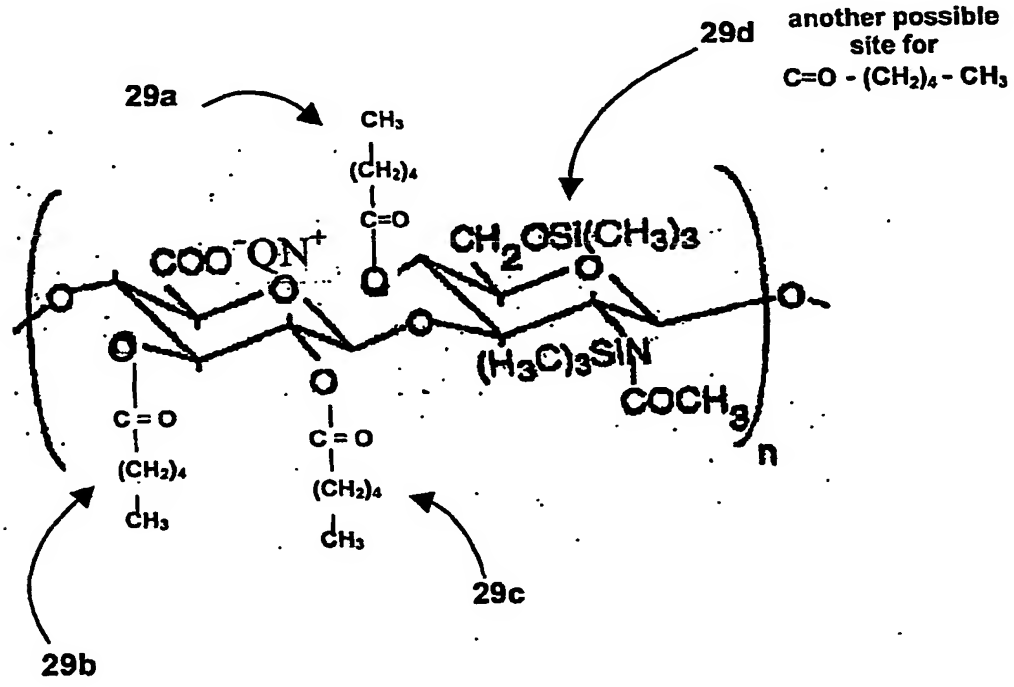


FIG. 2E

Depicts silylated HA^--QN^+ complex structure [*i.e.* H was replaced with $Si(CH_3)_3$] that has been acylated, with $Si(CH_3)_3$ groups now replaced with $COCH_3(CH_2)_4$ – as shown:



CONFIDENTIAL SUMMARY – FOR INTERNAL CSU USE ONLY

Graphs and Summary Table for:

ATTACHMENT A to Provisional Application CSURF-107P

HA Esterification via Acylation Technique for Moldable Devices

Internal TECHNICAL DISCLOSURE ADDENDUM

Min Zhang, Ph.D. candidate, Mechanical Engineering
Susan P. James, Ph.D. Associate Professor, Mechanical Engineering
Colorado State University

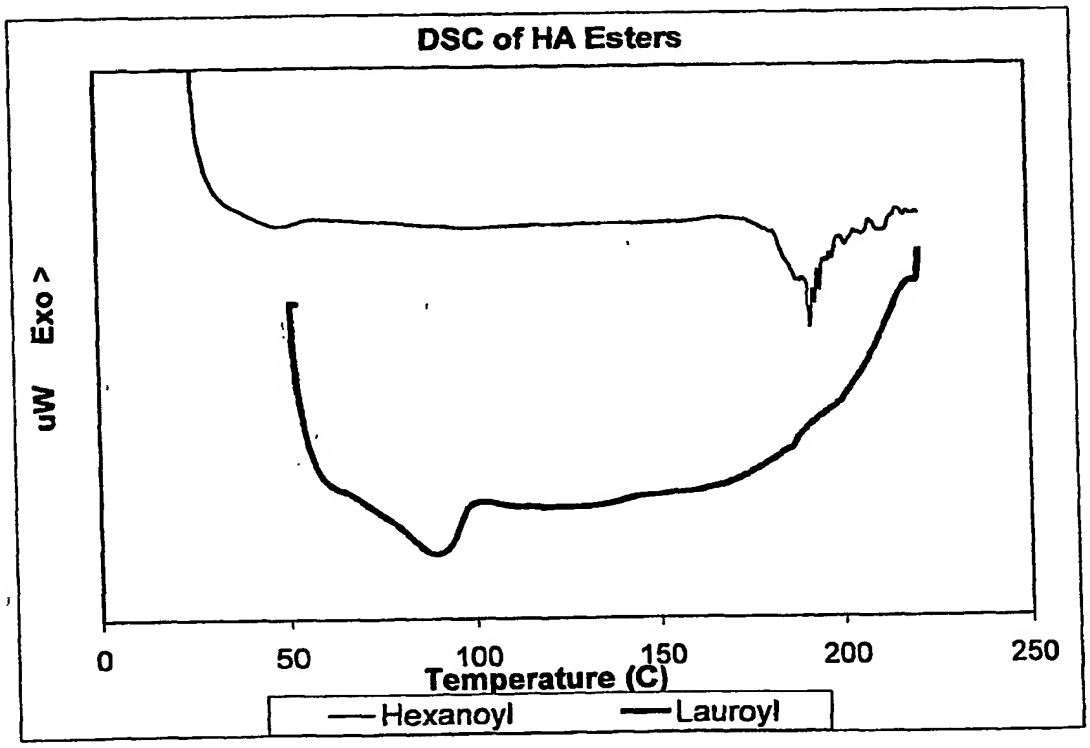


Figure 1 DSC of HA esters

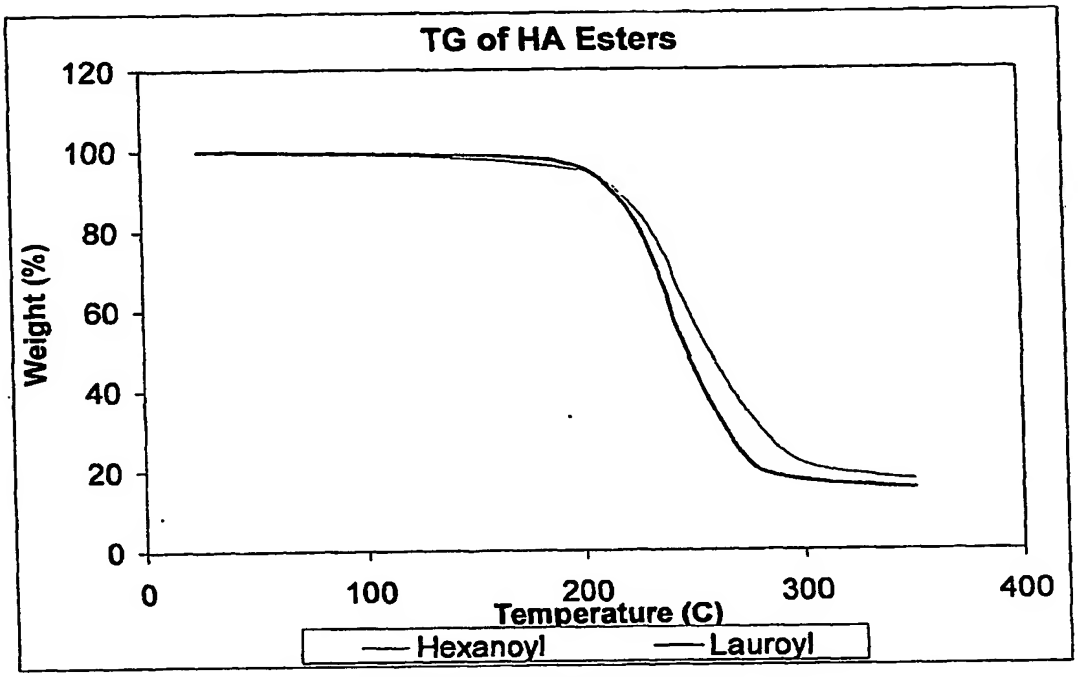


Figure 2 TGA of HA esters

--SUMMARY TABLE– THERMAL PROPERTIES OF HA ESTERS MADE WITH DIFFERENT ACYL RADICALS			
Acyl Radical	DSC Melting Range (Peak Endotherm), °C	TGA Onset of Degradation, ° C	First Visual Observation of Melting, ° C
Hexanoyl ($CH_3(CH_2)_4CO$-)	175-218 (190)	170	~190
Lauroyl ($CH_3(CH_2)_{10}CO$-)	70-105 (93)	190	100